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Molecular Definition of a Major Cytotoxic T-Lymphocyte Epitope in the Glycoprotein of Lymphocytic Choriomeningitis Virus†

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Analyses with segmental reassortants of lymphocytic choriomeningitis virus (LCMV) RNA have shown that cytotoxic T lymphocytes (CTL) are induced by and recognize proteins encoded by the viral short segment, which specifies two virus structural proteins, glycoprotein (GP) and nucleoprotein (NP). Expression of cDNA copies of these genes in vaccinia virus vectors demonstrates that C57BL/6 ($H2^{bb}$) mice mount significant CTL responses to both GP and NP. We have used LCMV-specific $H2^{bb}$-restricted CTL clones and a family of serial C-terminal truncations of the LCMV GP expressed in vaccinia virus to map the precise specificities of the anti-GP clones. Of the 18 CTL clones studied, 1 recognizes NP and the other 17 recognize GP. The reactivities of 14 of the 17 anti-GP CTL clones against the deleted GP molecules have been fully characterized, and two clear patterns of anti-GP activity have emerged, defining at least two CTL epitopes. The first epitope, recognized by only two of the clones, lies within GP residues 1 to 218. The second is recognized by all 12 of the remaining clones and was mapped, by using the GP deletions, to a 22-amino-acid region comprising GP residues 272 to 293. A synthetic peptide representing this area sensitized uninfected syngeneic target cells to lysis both by bulk CTL obtained from the spleen after a primary immunization and by appropriate CTL clones. Two sets of criteria are available which are said to identify potential T-cell epitopes, one based on primary amino acid sequence and the second based on protein secondary structure. Neither of these predictive schemes would have identified region 272 to 293 as a CTL recognition motif, indicating that such programs are of limited usefulness as presently conceived. Analysis of the CTL clones shows clearly that all three families (anti-NP and anti-GP 1 to 218 and 272 to 293) direct efficient cross-reactive killing against a variety of serologically distinct strains of LCMV.

The molecular basis by which a cytotoxic T lymphocyte (CTL) recognizes a virus-infected cell is interesting from two perspectives. First, for certain virus infections and tumors, CTL play a major role in limiting their spread through eradication of infected or transformed cells (14, 45). A detailed analysis of their function may allow their manipulation as therapeutic tools; adoptive transfer of this cell type has already been shown to terminate virus infection in experimental animals. Second, in a more general vein, CTL, through their receptors, recognize cells on the basis of how they present viral determinants restricted by the major histocompatibility complex (MHC) molecule(s) (17, 44). Understanding this trivalent recognition event (T-cell receptor–viral epitope–MHC) will allow us to approach the basis of the specificity of cell-cell recognition in terms of structure-function relationships. Furthermore, understanding this interaction will allow us to probe for mechanisms of tolerance and self-recognition, as well as to understand the function and diversity of MHC genes.

We have chosen to approach these issues by analyzing lymphocytic choriomeningitis virus (LCMV). CTL, which recognize viral antigens in association (usually) with class I and (uncommonly) with class II molecules of MHC, were first demonstrated in studies with LCMV (23, 44), as was their Thy1.2 phenotype (G. A. Cole, R. A. Prendergast, and C. S. Henney, Fed. Proc. 32:964, 1973) and their ability to cause injury in vitro (19, 27) and in vivo (12, 15). LCMV is capable of establishing a lifelong persistent infection in its natural host, the mouse, and CTL play a pivotal role in determining whether viral persistence will result from primary infection (reviewed in reference 9). LCMV infection of an immunocompetent adult mouse most often induces a brisk CTL response which eradicates the virus; the recovered animal is thereafter immune to further infection. In the absence of a CTL response, however (for example, if infection occurs in utero or in the neonatal period), the virus is not cleared and viral nucleic acid and protein can be detected in numerous organs throughout the life of the animal (37). Such a persistent infection can be terminated by reconstitution of such animals with Thy1.2$^+$ Lyt2$^+$ L3T4$^-$ immune memory cells (26).

LCMV, a member of the arenavirus family, has a bisegmented RNA genome encoding at least three polypeptides, one (the polymerase [35a]) on the long (L) RNA segment and two (the glycoprotein [GP] and nucleoprotein [NP]) on the short (S) segment (5, 30). On the $H2^{dd}$ background, proteins encoded on the S segment direct induction of, and target cell recognition and lysis by, CTL (31). The S segment of LCMV Armstrong (ARM) has been cloned and sequenced in its entirety (38) and encodes two proteins, a 558-amino-acid NP and a 498-amino-acid precursor GP, GP-C (7), which is posttranslationally cleaved to generate the two mature structural GP, GP-1 (residues 1 to 262) and GP-2 (residues 263 to 498) (8). Interstrain amino acid sequence comparisons of each of these molecules (36) have shown that the greatest degree of variability between different LCMV strains lies in GP-1, where the major antibody neutralization site has been identified (28). We have previously described (J. L. Whitton,

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P. J. Southern, and M. B. A. Oldstone, Virology, in press) the cloning and expression of a full-length NP and of a truncated GP (residues 1 to 363) and demonstrated that both of these moieties elicit a brisk primary CTL response in C57BL/6 ($H2^{bb}$) mice.

In the work described in this report, we analyzed the precise nature of the $H2^{bb}$ anti-GP CTL response by making a group of GP cDNA truncations expressed in recombinant vaccinia viruses (VV). Two CTL epitopes are defined, one being a short (22-amino-acid) sequence in LCMV GP-2 to which the major CTL response is mounted. The 22-amino-acid peptide was synthesized and used in CTL assays to demonstrate that it represents a cross-reactive epitope for other LCMV strains and that endogenous synthesis of viral antigens is not a requirement for CTL recognition.

MATERIALS AND METHODS

Cells, viruses and mouse strains. The growth medium for BALB/c C17 ($H2^{dd}$), HeLa, and 143TK$^-$ cells was minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 10^4 U of penicillin per ml, 10 mg of streptomycin per ml, 25 μg of amphotericin B per ml, and 1 mM L-glutamine. MC57 cells ($H2^{bb}$) were cultured in RPMI 1640 supplemented as above. All cell lines are mycoplasma free. The generation of the recombinant VV is described below. The origins of the five strains of LCMV used have been previously documented (13). C57BL/6 ($H2^{bb}$) and BALB/WEHI ($H2^{dd}$) mice were obtained from the breeding colony at the Research Institute of Scripps Clinic.

Construction of recombinant VV. Recombinant VV were made as described previously (20), with plasmid pSC11 as the intermediary vector (11). In brief, the desired LCMV coding sequences, all of which carry their own translational start and stop codons, were excised from the parental plasmids (see Results) and cloned into the *Sma*I site of pSC11, between the VV transcription initiation and termination signals. These materials were then introduced into VV by homologous recombination (20). A calcium phosphate precipitate of 10 μg of plasmid DNA was transfected into HeLa cells which had been infected 1 h previously with wild-type VV (multiplicity of infection [MOI], 0.1). After 48 h, virus was harvested and plated on 143TK$^-$ cells in the presence of 25 μg of bromodeoxyuridine per ml, allowing only TK$^-$ viruses to form plaques. To distinguish between recombinant VV and spontaneously occurring TK$^-$ mutants, the plaques were histochemically stained with 0.5% sterile agar–199 medium–300 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. Use of pSC11 as the cloning vehicle results in the cointroduction into the VV genome of the bacterial enzyme β-galactosidase, whose expression from recombinant virus causes the plaque to stain blue. Blue plaques were picked and plaque purified twice on 143TK$^-$ cells containing bromodeoxyuridine and twice more on HeLa cells without bromodeoxyuridine. Stocks of recombinant virus were prepared by infecting 143TK$^-$ cells at an MOI of 0.1, harvesting them 72 h postinfection, subjecting the collected cell mass to three cycles of freeze-thaw lysis (−70°C and 37°C), and treating it with 0.13% trypsin (15 min at 37°C) prior to storing it at −70°C in 100-μl aliquots.

The construction of VVnp and VVSC11 has been described previously (Whitton et al., in press). VVSC11, used in this paper in several cytotoxicity assays, is a recombinant VV which lacks any LCMV sequences but is β-galactosidase positive and TK$^-$.

Induction of bulk effector CTL. Primary LCMV-specific CTL were induced by intraperitoneal injection of 2×10^5 PFU of virus. Spleens were harvested at day 7 for all LCMV strains, except for LCMV PAST, which was obtained 8 to 9 days after inoculation. Primary anti-VV CTL were induced by intraperitoneal injection of 2×10^6 PFU of virus, with harvesting on day 6. Single-cell suspensions, free of erythrocytes, were used in subsequent cytotoxicity assays as described previously (10).

Isolation and maintenance of cloned CTL. LCMV ARM-specific CTL clones were generated from C57BL/6 mice ($H2^{bb}$) as previously described (10). Clones obtained by limiting-dilution techniques have been maintained for over 18 months. They are passaged weekly by culturing with LCMV ARM-infected syngeneic macrophages and syngeneic spleen cells (subjected to 2,000 rads of irradiation) in RPMI 1640 supplemented with 12% heat-inactivated calf serum, 1% glutamine, antibiotics, and 5% T-cell growth factor obtained by treating Lewis rat lymphocytes with concanavalin A, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, and 5×10^{-5} M β-mercaptoethanol. Once a week, autologous irradiated feeder cells are added, and medium is replaced three times a week. A total of 18 CTL clones were obtained from three independent experiments. The prefix describing each clone (RG-, HL 2-, or HL 3-) allows identification of the experiment from which it was derived. All clones were screened for LCMV ARM reactivity and $H2^{bb}$ restriction.

Cytotoxicity assays. Killing by cloned CTL and lymphocytes harvested from mouse spleens was quantitated with a ^{51}Cr release assay as described previously (10). Splenic lymphocytes were incubated with target cells at effector-to-target-cell ratios of 50:1 and 25:1, whereas CTL clones were incubated with target cells at effector-to-target-cell ratios of 5:1 and 2.5:1. Target cells were infected 48 h before the assay with LCMV at an MOI of 1 or infected with VV 6 h before the assay at an MOI of 3, or, for the peptide sensitization experiments, 40 μg of peptide was incubated with uninfected targets throughout the period of the assay. All samples were processed in triplicate, and standard deviation among triplicates was less than 5%. The percent specific ^{51}Cr release was calculated as 100 × (cpm sample release − cpm spontaneous release)/(cpm total release − cpm spontaneous release).

Peptide synthesis. The peptides described in the text were synthesized by the solid-phase method described by Merrifield (22) with an automated peptide synthesizer (no. 430A; Applied Biosystems, Foster City, Calif.). Peptide purity was assayed by high-pressure liquid chromatography and was greater than 80%.

Restriction enzymes and DNA sequencing. Restriction enzymes were purchased from several manufacturers and were used as specified by the manufacturers. DNA sequencing was carried out by using the chemical degradation technique of Maxam and Gilbert (21).

Analysis of LCMV-specific RNA from recombinant VV. Six 100-mm plates were seeded with 143TK$^-$ cells at 4×10^6 cells per plate and incubated overnight. The following day, one plate was mock-infected, and each of the remaining plates was infected at an MOI of 3 with one of the VV recombinants (VVa, VVc, VVd, VVe, or VVSC11). The plates were incubated for 5 h (after which time, marked cytopathic effect was present in all VV-infected cells), and total cytoplasmic RNA was harvested as follows. Minimal essential medium was aspirated, and the cells were washed three times in ice-cold phosphate-buffered saline. Ice-cold

isotonic lysis buffer (1 ml, containing 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 7.8], and 0.65% [vol/vol] Nonidet P-40) was added, and the cells were collected by scraping, transferred to a 1.5-ml Eppendorf vial, and incubated on ice for 10 min with occasional agitation. Following centrifugation at 3,500 rpm (1,000 × *g*) in an Eppendorf centrifuge, the supernatant was retained in a 15-ml polypropylene tube on ice, and the pellet (consisting of nuclei and debris) was suspended in a further 1 ml of ice-cold isotonic lysis buffer. After 10 min on ice the sample was spun as before, and the supernatant was collected and combined with the portion in the 15-ml tube. To this was added 2 ml of phenol extraction buffer (7 M urea, 350 mM NaCl, 10 mM EDTA, 10 mM Tris hydrochloride [pH 7.9], 1% [wt/vol] sodium dodecyl sulfate), and 4 ml of phenol-chloroform (1:1, vol/vol). This mixture was shaken vigorously before being centrifuged to separate the phases. The aqueous layer was retained, and 3 volumes of ethanol were added. The concentration and integrity of the RNA was determined by electrophoresis on a denaturing gel. For Northern (RNA) blot analysis, 10 μg of total RNA from each sample was run on a 1% agarose gel with 6% formaldehyde in morpholinepropanesulfonic acid (MOPS) buffer. Prior to electrophoresis, 2 μl of a 2-mg/ml solution of ethidium bromide was added to each sample; as a result, the RNA can be clearly visualized after electrophoresis without staining of the gel, allowing easy and accurate confirmation of the amount and intactness of the RNA in each track. The gel was blotted onto nitrocellulose overnight in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the filter was baked at 80°C for 2 h under vacuum. The blot was hybridized under standard conditions with 5 × 10^6 cpm of randomly labeled DNA probe; the LCMV ARM-specific probes used came from either the GP or the NP region of the genome (38) and were gel purified prior to being labeled.

RESULTS

Construction and expression of GP deletions in VV. The cloning of a truncated LCMV GP gene, encoding amino acid residues 1 to 363, has been described previously (Whitton et al., in press). The resultant plasmid contains the GP translation initiation codon and coding region up to residue 363, followed by 6 plasmid-encoded amino acid residues preceding a translation termination codon. This vector was used as the parent from which a family of serial GP truncations were made (Fig. 1). The parental plasmid was cleaved with restriction endonuclease *Xba*I, which cuts between the GP-coding sequences and the termination codon. The resultant linearized molecule was subsequently cut with either *Pst*I, *Tth*111I, or *Dra*III, which have unique cleavage sites within the GP-coding region. The DNA was then treated with the Klenow fragment of DNA polymerase I to generate blunt molecular termini and religated with T4 DNA ligase. This procedure yielded three serially deleted GP-coding regions, in all cases linked to the same plasmid sequences and termination codons. Figure 1 shows the nucleic acid and consequent amino acid sequences at the cloning junction of all three deleted molecules, as well as the sequences of the parental plasmid. The nucleic acid sequences shown were established by DNA sequencing of the plasmids. In all four plasmids, the DNA fragment containing the truncated GP sequences could be excised along with the translation termination codons by digestion with restriction enzymes *Bam*HI and *Hin*dIII. These fragments were then treated with Klenow fragment and deoxynucleoside triphosphates to blunt

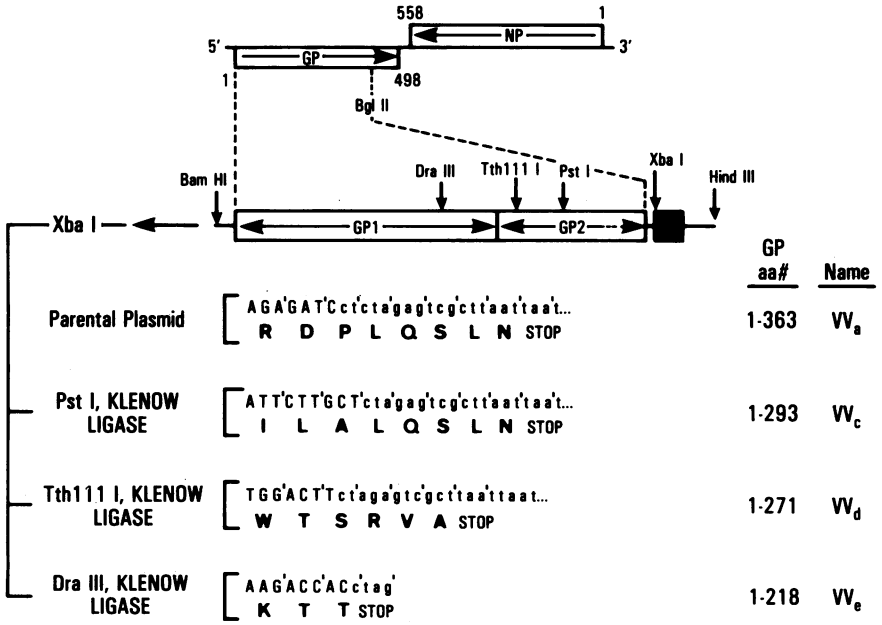
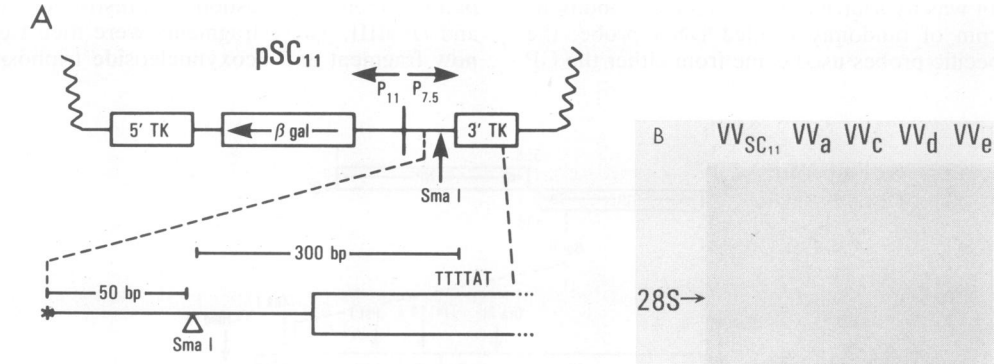


FIG. 1. Construction of the family of C-terminal truncations of LCMV GP. The LCMV S segment is shown, encoding GP and NP. – – –, location in the S segment of the *Bam*HI-*Bgl*II GP fragment used to make the parental plasmid. The construction of this plasmid is presented in expanded form; ■, universal translation terminator sequence (see Materials and Methods). Restriction enzyme sites used to make the deletions are shown above the expanded area. DNA sequences at the cloning junctions were confirmed by sequencing and are shown; capital letters represent GP-derived nucleotides; lowercase letters represent plasmid-derived bases. The vertical dashes above the DNA sequences indicate the open reading frame from GP, and the encoded amino acids are shown below the DNA sequences in single-letter code. The names of the resultant VV recombinants and the number of LCMV GP residues encoded therein are shown (aa, amino acid).

the termini and cloned into the *Sma*I site of plasmid pSC11 (11) (Fig. 2A). This plasmid encodes the bacterial β-galactosidase gene under the control of a VV promoter, allowing selection of virus recombinants by blue plaque morphology (see Materials and Methods). We next tested the recombinant VV for expression of the sequences we had introduced. The *Sma*I site of pSC11 lies some 50 base pairs downstream of the transcription start site and approximately 300 base pairs upstream of the 3′ end, which is defined by transcription termination signals in the TK gene sequences (Fig. 2A). As a result, the length of the mRNA which encodes the sequences inserted into the *Sma*I site is the sum of four features: 50 bases at the 5′ end, the insert, 300 bases at the 3′ end, and the poly(A) tail (which is added by a VV-encoded enzyme and may be several hundred residues long [25, 32]). The expected sizes [before addition of poly(A)] of the four GP mRNAs expressed from VV are shown in Fig. 2A. Cytoplasmic RNA was recovered from cells 4 h after infection with the VV recombinants, subjected to electrophoresis, and blotted onto nitrocellulose. A GP-specific DNA probe was used to identify the VV-encoded LCMV-specific RNA molecules. The resulting bands (Fig. 2B) are of approximately the expected sizes, and no other bands are noticeable, suggesting that neither premature transcriptional termination nor readthrough beyond the VV TK gene termination site occurs. Analysis of protein expression by immunofluorescence techniques indicated that each virus recombinant was expressing the encoded LCMV polypeptide (data not shown).

LCMV-specific H2-restricted recognition of recombinant VV. We next wished to see whether CTL induced by LCMV ARM were able to recognize and lyse cells infected with the recombinant VV. We have previously demonstrated that VV_{np} is recognized (Whitton et al., in press), and the data presented in Fig. 3 deal with the recombinant VV containing the variety of LCMV GP truncations. The results show that bulk splenocytes from a C57BL/6 mouse infected 7 days previously with LCMV ARM were able to lyse syngeneic targets infected with each of the four recombinant viruses which express LCMV GP. This lysis was LCMV specific, since cells infected with wild-type VV (VVwt) were not lysed (although these cells were productively infected and were efficiently lysed by anti-VV CTL), and H2 restricted, since the $H2^{bb}$ bulk CTL did not lyse allogeneic targets infected with LCMV ARM and allogeneic effector cells did not lyse the $H2^{bb}$ targets infected with recombinant VV. Thus, all of the GP recombinant viruses express LCMV determinants in a manner allowing CTL recognition and lysis. Recognition of VVe indicates that at least one CTL epitope must lie in GP residues 1 to 218. Of the four GP recombinants, three (VVa, VVc, and VVd) encode a full-length mature LCMV protein (all three contain the full coding region of GP-1). However, VVe encodes only a partial copy of GP-1; the fact that it is efficiently lysed shows that native protein is not required for CTL recognition and lysis.

Seventeen of eighteen CTL clones recognize the LCMV GP moiety. Eighteen independently derived CTL clones were



Recombinant Vaccinia	LCMV Protein Encoded	Length of DNA Insert (bp)	Expected VV mRNA Size
VV_a	GP 1-363	1200	1550
VV_c	GP 1-293	1000	1350
VV_d	GP 1-271	930	1280
VV_e	GP 1-218	770	1120

FIG. 2. Transcriptional strategy of plasmid pSC11, used to predict the approximate lengths of VV-LCMV recombinant mRNA molecules in the recombinant VV. The layout of pSC11 is shown. Abbreviations: 5′ TK and 3′ TK, 5′ and 3′ portions of the VV thymidine kinase gene, respectively; β gal, gene encoding β-galactosidase; P_{11} and $P_{7.5}$, two divergently oriented VV promoters; bp, base pair. The insertion site for LCMV DNA is shown by an arrow. The asterisk indicates the 5′ end of the mRNA initiated on the $P_{7.5}$ promoter, and TTTTAT is the transcription terminator in the 3′ TK region. The rationale behind the calculations in the lower half of the figure is explained in Materials and Methods. Note that the right-hand does not include the poly(A) tail, which may be some 200 to 400 bases in length. (B) Northern blot analysis of the four VV GP recombinants. Samples of total cytoplasmic RNA from cells infected with VV_{11}, VVa, VVc, VVd, and VVe were electrophoresed and transferred to nitrocellulose as described in Materials and Methods. The blot was hybridized with a probe specific for LCMV GP, washed, and exposed at −70°C to Kodak XAR5 film with a Cronex Lightning-Plus (du Pont) intensifying screen. The positions of the 28S rRNA (ca. 5,200 bases) and the 18S rRNA (ca. 2,000 bases) are shown by arrows.

used to analyze the effects of the GP deletions on CTL recognition and lysis. The results of these analyses are presented in Fig. 4. Seventeen of the clones lyse VVa-infected cells and therefore recognize an epitope or epitopes entirely contained in GP residues 1 to 363. These results are striking in the preponderance of GP recognition. In, for example, the influenza virus system, the majority of CTL recognition is directed toward internal proteins (NP, NS1, and the polymerase complex); however, in the LCMV natural infection, none of 18 CTL clones are directed toward polymerase, and only 1, HL 2-1, recognizes NP. Although at this stage we cannot exclude the possibility that some degree of selection in favor of certain clones has occurred during the cloning procedure, these results are consistent with our earlier observations that a significant part of the primary CTL response was against the GP moiety.

GP-specific CTL clones comprise at least two epitope-specific families. Further analysis (Fig. 4) of the 17 CTL clones which recognize the LCMV GP moiety shows that they fall cleanly into at least two families. Two of the clones (RG-1 and HL 2-2-3) lyse syngeneic target cells infected with all four GP recombinants. These clones therefore recognize the epitope (or epitopes) inferred above, which are contained in GP residues 1 to 218. Of the remaining 15 clones, 12 have been tested against all four recombinants, and all of these fall into a group which recognizes VVa and VVc, but not VVd or VVe. Clone RG-2 also may be in this group, since it recognizes VVa- but not VVd- or VVe-infected cells; however, it has not been tested against VVc. The final two clones have been tested only against VVa. Thus, at least 66% (and perhaps as many as 85%) of the CTL clones are in a family which recognizes VVc (GP residues 1 to 293) but fails to lyse VVd (GP residues 1 to 271). Therefore, amino acids 272 to 293 are required to allow CTL recognition by these clones. This region is near the N terminus of GP2. Note that although some of the CTL clones exhibit a fairly high level of background lysis (e.g., HL 2-2-1 and HL 3-9), even in these cases there is a very clear discrimination between the level of lysis of VVa and VVc compared with that of VVd, VVe, VVwt-VVSC11, and uninfected cells. The high level of

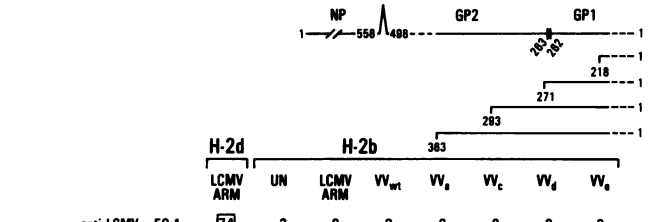


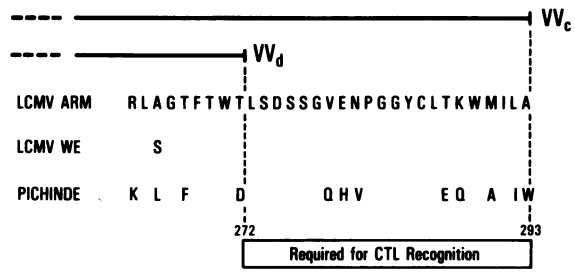
FIG. 3. CTL recognition of LCMV GP truncations. All four truncated LCMV GP molecules expressed are recognized by $H2^{bb}$ bulk splenocytes. Preparation of effector and target cells and the cytotoxicity assay were as described in Materials and Methods. In this and all subsequent figures presenting data from such assays, the effector cells are listed to the left of the data and the target cells are indicated above the data. The percent specific ^{51}Cr release considered significantly above background is boxed. The extent of each GP truncation is delineated above the data.

		H2b							H2d
		UN	LCM ARM	VV$_{wt}$ or VV$_{SC_{11}}$	VV$_a$	VV$_c$	VV$_d$	VV$_e$	VV$_{NP}$	LCM ARM
H2b	anti LCM ARM	4 1	55 39	5 1	48 28	57 42	56 32	39 21		
H2b CTL Clones	RG 1	10 4	39 28	12 10	41 31	44 38	42 36	42 34		15 8
	HL 2-2-3	0 0	48 34	4 3	46 36	48 39	48 36	46 36		7 6
	RG 4	16 11	78 65	14 10	70 62	53 46	17 14	12 10		20 12
	RG 9	9 5	75 65	16 9	64 55	68 59	24 16	21 15		4 3
	RG 16	22 16	89 80	19 13	82 76	68 60	16	12		22 15
	RG 17	7 3	84 71	6 3	85 73	78 72	10 2	6 4		14 4
	HL 2-2-1	51 42	82 71	48 36	75 68	68 60	40 37	38 29		30 17
	HL 2-2-8	26 78	83 78	19 13	74 62	74 72	23 16	16 11		5 5
	HL 2-2-9	23 13	77 70	18 11	68 56	65 60	16 10	17 10		12 6
	HL 2-3-2	6 4	74 62	3 2	63 51	70 66	2 0	3 2		8 2
	HL 3-1	4 5	59 44	3 3	47 40	46 45	8 2	4 2		10 5
	HL 3-2	15 9	93 85	9 7	82 72	68 74	21 15	16 13		0 3
	HL 3-7	55 47	89 83	53 47	85 73	88 81	65 56	54 44		14 8
	HL 3-9	50 38	85 86	54 39	78 82	86 82	56 50	51 45		84 83
	RG 2	15 12	77 69	14 10	72 67		17 16	12 7		34 21
	HL 2-18	0 1	49 38	0 0	40 28					
	HL 3-17	33 20	78 66	23 12	68 66					
	HL 2-1	20 15	77 75	23 19	20 18	23 23	30 24	24 20	68 51	12 7
H2d	anti LCM ARM									74 63

FIG. 4. Analysis of the specificities of 18 CTL clones against the VV GP recombinants. The cytotoxicity assay was carried out as described in the text; for each CTL clone two effector-to-target-cell ratios were analyzed: 5:1 and 2.5:1. The percent specific lysis considered significantly above background is boxed.

killing by HL 3-9 of the $H2^{dd}$ cells may reflect alloreactive lysis; we have not fully analyzed this clone, but another isolate from the same round of CTL cloning is $H2^{bb}$ restricted for LCMV and also alloreactive against uninfected $H2^{dd}$ cells (unpublished data).

Use of synthetic peptides to confirm that GP 272-293 contains a CTL epitope. The demonstration that GP residues 272 to 293 are required to allow target cell recognition and lysis could be interpreted in three ways. The simplest explanation is that the region contains the CTL epitope. Alternatively, the epitope may span residues 271 to 272 and be destroyed by the deletion. Finally, the epitope may lie outside this region, but this region may be needed to allow, for example, correct processing or presentation of the epitope. The most direct way of testing these hypotheses was to make a synthetic peptide to this region and to analyze the ability of this molecule to confer upon syngeneic (uninfected) target cells the ability to be recognized and lysed by CTL. The amino acid sequences of this region in two different LCMV strains and in another arenavirus, Pichinde virus, are shown



				H2bb UN	H2bb LCMV ARM	H2bb 272-293 UN	H2bb HLA UN
H2bb Bulk Splenocytes	anti ARM	50:1		14	[84]	[28]	12
		25:1		11	[82]	[25]	11
α GP	HL 2-2-8	5:1		6	[57]	[31]	6
		2.5:1		2	[49]	[27]	1
α GP	HL 2-32	5:1		2	[79]	[41]	8
		2.5:1		2	[60]	[18]	5
αNP	HL 2-1	5:1		7	[64]	14	18
		2.5:1		5	[46]	10	17

FIG. 5. CTL recognize synthetic peptide. A synthetic peptide representing GP residues 272 to 293 appropriately directs killing of uninfected syngeneic target cells. The amino acid sequences of LCMV ARM (38) and WE (33) and of Pichinde arenavirus (33) are illustrated in single-letter code. The GP-coding boundaries of VVc and VVd are indicated, as is the 22-amino-acid region required for CTL killing (see Fig. 4 and text). This region was synthesized as a peptide and used in a cytotoxicity assay, the results of which are presented in the lower half of the figure. The effector cells used are shown: both of the anti-GP clones used were VVc^+ VVe^- (Fig. 4). The target cells were uninfected, infected with LCMV ARM, and uninfected and treated with 40 μg of either GP residues 272 to 293 or an irrelevant peptide human lymphocyte antigen. The percent ^{51}Cr release considered significantly above background is boxed.

in Fig. 5. A synthetic peptide representing residues 272 to 293 of LCMV ARM was synthesized and used to sensitize target cells to CTL killing (see Materials and Methods). The effect of the peptide was tested by coincubation with bulk LCMV CTL and with three CTL clones, HL 2-2-8, HL 2-32 (both of which recognize VVc but not VVe), and HL 2-1, which is NP specific. Both HL 2-2-8 and HL 2-32 lyse cells treated with this peptide, as do bulk CTL, although splenic CTL are less efficient. The specificity of the effect of this peptide is seen in the failure of these effector cells to lyse cells treated with an irrelevant peptide (a peptide from human lymphocyte antigen B27). The specificity of the two CTL clones HL 2-2-8 and HL 2-32 is reconfirmed by the inability of clone HL 2-1 to lyse targets treated with the peptide from residues 272 to 293. These findings show conclusively that residues 272 to 293 contain a CTL epitope (or epitopes) and confirm the validity of using serially truncated molecules expressed from VV to map CTL epitopes.

Both GP and NP epitopes direct cross-reactive CTL recognition and lysis. Finally, we wished to analyze the cross-reactive CTL response in LCMV infection. Current dogma, established from studies on the influenza virus and vesicular stomatitis virus animal model systems, suggests that almost all cross-reactive CTL (that is, CTL induced by one virus serotype but able to recognize a different serotype) are directed against internal proteins such as NP and polymerase moieties (2, 4, 29, 43); anti-glycoprotein CTL are serotype

				H2bb ARM	H2bb PAST	H2bb TRAUB	H2bb WE	H2bb UN
Bulk Splenocytes	$H2^{dd}$	anti ARM	50:1	0	0	1	12	6
	$H2^{bb}$	UNINF	50:1	3	0	0	7	0
	$H2^{bb}$	anti ARM	50:1	[38]	[19]	[37]	[36]	1
$H2^{bb}$ CTL Clones	α 1-218	HL2-2-3	2.5:1	[33]	[37]	[54]	[59]	7
	α 272-293	HL 3-1	2.5:1	[65]	[48]	[64]	[78]	5
	α NP	HL2-1	2.5:1	[68]	[68]	[60]	[60]	36

FIG. 6. Killing of other strains of LCMV by anti-LCMV ARM bulk splenocytes and CTL clones against three discrete LCMV ARM epitopes. The cytotoxicity assay was carried out as described in the text, but for these data only one effector-to-target-cell ratio was used for each sample. The specificity of each of the three CTL clones, as far as has been determined by present results, is shown to the left of the clone name.

specific. However, we have previously noted that in the natural infection by LCMV, it is possible to detect a major cross-reactive CTL response against GP, and these observations are expanded in Fig. 6 and 7.

The cross-reactivity of anti-LCMV ARM CTL with LCMV PAST, Traub, and WE has been previously documented (1) and is confirmed in Fig. 6; however, we also show that CTL clones directed against GP residues 1 to 218 (HL 2-2-3), GP residues 272 to 293 (HL 3-1), and NP (HL 2-1) are cross-reactive with these other LCMV strains. We thus extend our previous results in showing that in agreement with results from other systems, cross-reactive CTL can be elicited by internal proteins (in this case, LCMV NP). Furthermore, we show that the cross-reactive anti-GP response we previously described is induced by at least two entirely distinct GP epitopes, encompassing residues 1 to 218 and 272 to 293.

The precise mapping of the GP-2 epitope to residues 272 to 293 allows us to predict, from the sequence comparison in Fig. 5, that CTL clones against the GP region from residues 272 to 293 would cross-react with LCMV WE, which is identical in sequence in this region; in contrast, such CTL clones would probably fail to cross-react with Pichinde

				H2bb ARM	H2bb 1-293 VV_c	H2bb 1-218 VV_e	H2bb $VV_{SC_{11}}$	H2dd ARM
$H2^{bb}$ Bulk Splenocytes	anti ARM	50:1		[62]	[37]	[30]	6	13
		25:1		[45]	[28]	[22]	6	8
	anti PAST	50:1		[52]	[30]	[30]	7	7
		25:1		[26]	[19]	[20]	5	6
	anti TRAUB	50:1		[21]	[18]	[20]	3	5
		25:1		[13]	[10]	[14]	2	5
	anti E350	50:1		[71]	[71]	[47]	6	7
		25:1		[46]	[28]	[25]	5	4
	UNINF	50:1		0	4	3	3	3
		25:1		0	2	2	4	3
$H2^{dd}$	anti ARM	50:1		0	3	3	3	[62]
		25:1		0	1	3	3	[31]

FIG. 7. Anti-GP cross-reactive CTL. CTL induced by three other LCMV strains efficiently lyse target cells infected with recombinant VV expressing LCMV ARM GP. Effector cells were induced by infection of $H2^{bb}$ mice with the four LCMV strains shown and harvested as described in Materials and Methods. The cytotoxicity assay was carried out as described in the text, and the percent specific ^{51}Cr release considered significantly above background is boxed.

virus, whose sequence is very different. These predictions are fulfilled, since Pichinde virus is not recognized by any of the 18 CTL clones (data not shown), whereas clone HL 3-1 does indeed recognize strain WE. This clone also recognizes the PAST and Traub strains, suggesting that in these two strains major sequence changes in this region are unlikely. We have no fine mapping information regarding the cross-reactive epitopes in GP residues 1 to 218 or in NP.

Thus, three other strains of LCMV allow cross-reactivity when they are used as target antigens for anti-ARM CTL clones. We next wished to ask whether the cross-reactivity was reciprocal; that is, whether alternative strains of LCMV induce CTL which can cross-react with the LCMV ARM GP. CTL induced by strains PAST, Traub, and E350 all can cross-react with both VVc and VVe constructs (Fig. 7). These data indicate that an epitope (or epitopes) within ARM GP residues 1 to 218 can be seen by cross-reactive CTL; however, since this postulated epitope is also present in VVc, it is not possible from these results to determine whether the lysis of VVc-infected cells is due to recognition of the epitope within residues 1 to 218 or perhaps that within residues 272 to 293. This question can be addressed in the future by using the synthetic peptide technique.

DISCUSSION

The studies we present involve the use of serially truncated GP molecules and synthetic peptides to sensitize target cells to CTL killing. The results relate to the broader aspects of CTL recognition and MHC presentation, as well as helping define the immunobiology of LCMV. The precise nature of a class I-restricted CTL epitope and the requirements for its successful in vivo presentation remain unclear. Certain differences between class I and class II restriction have been proposed and are addressed by our studies. First, it has been suggested that whereas class II molecules restrict processed antigen, class I molecules instead restrict intact (native) antigen. LCMV CTL are class I restricted (44, 45), and the recognition of VVe by splenic CTL generated after primary inoculation and by CTL clones RG-1 and HL 2-2-3 clearly demonstrates that native LCMV protein cannot be required for CTL recognition and lysis, since VVe encodes only a truncated GP-1 (residues 1 to 218). Furthermore, the epitope(s) near the N terminus of GP-2 is seen by LCMV-specific CTL clones, when presented either as a peptide (Fig. 5) or by VVc, which encodes only residues 263 to 293 of the GP-2 moiety. Thus, native GP-2 also is not required for successful presentation of this epitope(s). Together these findings show that native protein is not a prerequisite for recognition of class I-restricted antigens. Moreover, the VVc recombinant that expresses GP-2 residues 263 to 293 also contains five non-LCMV amino acids attached to residue 293 (Fig. 1). Despite these foreign components, VVc is efficiently recognized by the CTL clones. It is unclear whether this region is processed during VVc infection to remove the non-LCMV amino acids prior to presentation by the MHC, or whether the T-cell receptor of the CTL clone is able to recognize the presented peptide despite these five accessory residues. Whichever is the case, the recognition of this CTL epitope in VVc attests to the flexibility of the system, which allows effective presentation and recognition of an epitope despite the nearby covalent attachment of several foreign amino acids. These observations support the concept that class I MHC restriction may be similar to class II restriction in the presentation of small processed regions of virus polypeptide (35, 42). Our studies of the CTL interaction with LCMV GP complement and extend the initial observations of Townsend et al., who studied transfected deletion mutants of influenza virus nucleoprotein (40) and an influenza hemagglutinin apparently lacking cell membrane expression (39).

It has also been suggested that class I alleles restrict only endogenously synthesized molecules, whereas class II alleles restrict only exogenously applied molecules (24). Our observation that a peptide along with a syngeneic uninfected target cell can direct CTL killing confirms studies by Townsend et al. of NP peptide in the influenza virus system (41). These observations, as well as refuting any requirement for native antigen in CTL recognition, indicate that endogenous synthesis is not required to sensitize target cells to CTL recognition and lysis in vitro. However several important questions about the mode of action of the peptide in vitro remain unanswered. For example, does the peptide bind directly to the MHC molecule on the cell surface or is it first internalized by the presenting cell? If it is internalized, is it subjected to processing prior to presentation? Thus, although the results obtained with the peptide confirm the presence of at least one epitope in GP residues 272 to 293, our data do not disprove an in vivo requirement for endogenous synthesis of class I-restricted antigens.

Attempts have been made to devise methods by which T-cell epitopes could be predicted from primary amino-acid sequences. The presence of an amphipathic helix has been suggested as an important component of helper T-cell (MHC class II-restricted) epitopes (16); however, the region from residues 272 to 293, which we show contains a major class-I restricted epitope, does not comprise an amphipathic helix. An alternative prediction (34), made by comparing the primary sequences of a variety of class I- and class II-restricted epitopes, suggests that a T-cell epitope comprises an amino acid tetramer (C or G)-H-H-(C or P) (where C is a charged residue, H is a hydrophobic residue, P is a polar residue, and G is glycine). Although this has been used to predict at least one NP epitope of influenza virus (3), no such tetramer exists in the 22-amino-acid stretch defined by our peptide. Therefore, neither of the two currently used prediction schemes designed to identify T-cell epitopes would have accurately located this major virus CTL epitope. Clearly, more data characterizing linear epitopes are required before reliable guidelines can be developed.

A number of generalizations about the overall pattern of CTL responses to MHC class I-restricted viral antigens that have been suggested from studies of influenza A virus (4, 18), vesicular stomatitis virus (29), and respiratory syncytial virus (2) must be modified as a result of our data with LCMV. First, although a few conflicting reports have occurred (6, 43), the current view is that the major overall CTL responses to influenza virus, respiratory syncytial virus, and vesicular stomatitis virus are directed against nonglycosylated proteins; the response to viral GP frequently has been shown to be low level (29) or undetectable (2). Our observations indicate that with LCMV infection on the C57BL/6 $H2^{bb}$ background, a major primary CTL response is mounted against the viral GP. The extent of the anti-GP primary response is reflected in the efficient lysis by $H2^{bb}$ splenic lymphocytes taken from primed mice and reacted with syngeneic cells infected with any of the VV GP recombinants (Fig. 3). In this report we show that of 18 independently isolated CTL clones obtained, 17 are directed against the GP molecule (and the majority of these are directed against a discrete region at the N terminus of GP-2). Although it is possible that some degree of selection occurred

during the cloning manipulations, the overwhelming anti-GP response is compatible with the results obtained when using bulk splenocyte CTL populations. The second point is in regard to cross-reactive CTL responses. With the LCMV model (Fig. 6 and 7; Whitton et al., in press) it is clear that the major anti-GP response is cross-reactive, in contrast to the influenza virus, vesicular stomatitis virus, and respiratory syncytial virus systems, in which the cross-reactive CTL response is directed mainly against internal viral proteins. For example, CTL clones directed against two distinct regions of the GP of LCMV ARM can lyse target cells infected with three other virus strains, PAST, Traub, and WE (Fig. 6). Additionally, effector cells induced by three other strains (PAST, Traub, and E350) directly lyse targets expressing only the ARM strain GP (Fig. 7); CTL induced by these LCMV strains recognize an LCMV ARM epitope(s) between GP residues 1 and 218, since these effectors lyse H2-restricted targets infected with VVe. These results amply demonstrate that anti-GP CTL can play a major role in cross-reactive CTL recognition, although internal antigens can also direct cross-reactive killing in the LCMV system. For example, clone HL 2-1, which was induced by LCMV ARM and is anti-NP (Fig. 4), can lyse syngeneic targets infected with three serologically distinct LCMV strains (Fig. 6). Thus, both GP and NP can induce cross-reactive CTL responses. Our findings with C57BL/6 ($H2^{bb}$ mice) indicate that there are at least two viral GP epitopes and that the major one resides at GP-2 residues 272 to 293, whereas the major antibody neutralization epitope for LCMV is distinct from this epitope, since it lies on GP-1 (28). The divergence of these immunologically important sites points to the difficulty of utilizing a single-subunit vaccine for control of infection, but highlights the options and vigor of the immune system for responding to a microorganism. The use of truncated viral genes expressed in several H2 backgrounds should provide systematic data for molecular identification and definition of such sites on viral proteins and hence provide information for both therapeutic manipulation of the immune response and analysis of cell-cell interactions.

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